

Higher Muscle Content of Perilipin 5 and Endothelial Lipase Protein in Trained Than Untrained Middle-Aged Men

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Summary

A high VO₂max in middle-age is related to high metabolic flexibility and lowered risk of metabolic diseases. However, the influence of a high VO₂max induced by years of regular training in middle-age on protein expression related to muscle metabolism is not well studied. This study measures key proteins involved in mitochondrial oxidation, glucose and lipid metabolism in skeletal muscle of trained and untrained middle-aged men. 16 middle-aged men, matched for lean body mass, were recruited into an endurance trained (TR, n=8) or an untrained (CON, n=8) group based on their VO₂max. A muscle biopsy was obtained from *m. vastus lateralis* and protein levels were analyzed by Western blotting. The TR had higher protein levels of mitochondrial complex III-V, endothelial lipase (EL) and perilipin 5 compared to the CON. Glycogen synthase (P=0.05), perilipin 3 (P=0.09) and ATGL (P=0.09) tended to be higher in TR than CON, but there was no difference in AKT I/II, HKII, GLUT4 and LPL protein expression. Lastly, there was a positive correlation between plasma HDL and EL (R²=0.53, P<0.01). In conclusion, a high VO₂max in middle-aged men was as expected is reflected in higher muscle oxidative capacity, but also in higher endothelial lipase and perilipin 5 expression and a borderline higher glycogen synthase protein expression, which may contribute to a higher metabolic flexibility.

Key words

Lipid metabolism • Glucose metabolism • Endurance training • Middle-aged men • Human skeletal muscle

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Introduction

Having a high aerobic capacity (VO₂max) while we age contributes positively to muscle metabolic flexibility and decreases the age-related risk of developing metabolic diseases (Bonadonna *et al.* 1994, Kelley *et al.* 2002, Kelley and Mandarino 2000, Ritz *et al.* 1998, Storlien *et al.* 2004). At the muscular level a higher VO₂max is reflected in higher mitochondrial oxidative capacity, which is due to both increased mitochondrial mass and increases in mitochondrial complexes (Gram *et al.* 2014). However, the molecular mechanisms that control and regulate glucose uptake as well as lipid uptake and storage and thus affects metabolic flexibility are only partly understood.

In regards to glucose metabolism, a high VO₂max in both young (Dela 1996) and older men (Cox *et al.* 1999, Tonino 1989) is associated with an improved insulin-stimulated response and hence improved glucose metabolism. At the muscle level this has been coupled to increased protein levels of hexokinase II (Frosig *et al.* 2007), AKT I and II (Frosig *et al.* 2007), and GLUT4 (Cox *et al.* 1999, Dela *et al.* 1993, Hickner *et al.* 1997, Ren *et al.* 1994) in young individuals. But these proteins have, to our knowledge, not been analyzed together in the same study in middle-aged men before; such knowledge would provide information about the overall training induced differences in this age-group.

Fatty acid (FA) oxidation is improved by endurance training (Henriksson 1977, Hurley *et al.* 1986, Martin *et al.* 1993). At the muscular level this occurs through several adaptations. In FA uptake, endothelial lipase (EL) and lipoprotein lipase (LPL) are the primary

lipases involved in hydrolysis of triglycerides in plasma lipoproteins (Griffon *et al.* 2006, McCoy *et al.* 2002). LPL has been shown to increase with training in young individuals (Lithell *et al.* 1979, Nikkila *et al.* 1978, Taskinen and Nikkila 1980), but less is known about EL. EL is not expressed in muscle cells but in the endothelial cells and macrophages (Annema and Tietge 2011) and there is no evidence for a specific functional role of EL in skeletal muscle. However, based on a study of fat cells from mice that lacked LPL, it was demonstrated that EL mediates an alternative pathway for FA uptake in adipose tissue (Kratky *et al.* 2005). We have previously reported that EL expression in human skeletal muscle was similar between untrained healthy middle aged men and age and lean body mass matched men with type 2 diabetes or impaired glucose tolerance (Vigelsø *et al.* 2013). Yet, it is not known if EL expression in the endothelial tissue of human skeletal muscle is influenced by a marked difference in VO_2max after years of regular training.

The intramuscular release of FA from the lipid droplets for oxidation are controlled by hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), which are regulated by the lipid droplet related proteins, perilipin 2, 3 and 5 (Bartz *et al.* 2007, Brasaemle *et al.* 2004). In young individuals training increases the protein expression of perilipin 2 (Shaw *et al.* 2012, Shepherd *et al.* 2013), perilipin 3 (Louche *et al.* 2013) and perilipin 5 (Peters *et al.* 2012, Shepherd *et al.* 2013) in skeletal muscle. Although the regulation of lipid droplet function and muscle lipid uptake has improved considerably in the latter years this knowledge has primarily been gained from studies of young individuals and thus little is known about the effect of age and the influence of training. It is thus interesting to investigate differences in the key proteins in lipid uptake and lipid droplet regulation in aged individuals that exhibit a marked difference in maximal oxygen uptake through exposure to life-long training.

Therefore, we studied the expression of proteins involved in glucose and lipid metabolism in skeletal muscle from middle-aged men having either a normal or a high VO_2max . We hypothesized that middle-aged men with a high VO_2max would have higher levels of the key proteins involved in glucose uptake and lipid uptake and storage compared to untrained middle-aged men.

Material and Methods

Participants

16 middle-aged men were recruited into two

groups of either healthy sedentary controls (CON, n=8) or endurance-trained participants (TR, n=8). The groups were matched for age and LBM (Table 1). The participants in CON were not allowed to do regular exercise, whereas the TR group should perform regular endurance training (e.g. running or cycling). Furthermore, the participants in TR had all been performing regular endurance training for many years. Exclusion criteria were: prescribed medication, family history of type 2 diabetes, severe obesity or cardiovascular disease. Furthermore, the participants were instructed to refrain from alcohol, tobacco and strenuous exercise 48 h prior to the screening and experimental day. All participants received written and verbal information about the purpose and potential risks and complications of the experiment. Informed written consent was obtained before inclusion. The study was approved by the Copenhagen Ethics Committee (KF 01-091/02) and was carried out in accordance with the Declaration of Helsinki II.

Table 1. Group characteristics.

	Healthy sedentary controls (CON)	Endurance trained (TR)
N	8	8
#Age (years)	53 ± 2	51 ± 2
#Weight (kg)	93 ± 3	82 ± 5*
#BMI (kg·m ⁻²)	29 ± 1	25 ± 1*
#Whole body fat (%)	26 ± 1	18 ± 1*
#LBM (kg)	65 ± 2	63 ± 3
# VO_2max (ml O ₂ ·kg LBM ⁻¹ ·min ⁻¹)	43 ± 2	58 ± 2*
#ssGIR (mg·[kg LBM] ⁻¹ ·min ⁻¹)	13 ± 1	18 ± 1*
#Glycogen (nmol·mg ⁻¹ d.w.)	57 ± 7	107 ± 11*
IMTG (nmol·mg ⁻¹ d.w.)	33 ± 9	29 ± 9

Data are means ± SEM. BMI, Body mass index; LBM, Lean body mass; VO_2max , Peak oxygen uptake; ssGIR, steady state glucose infusion rate during the euglycemic-hyperinsulinemic clamp step 2. # Data have previously been published (Skov-Jensen *et al.* 2007, Skovbro *et al.* 2008, Vigelsø *et al.* 2013). * P<0.05

The present project was part of a large study that focused on insulin sensitivity and lipid metabolism in patients with type 2 diabetes or matched participants with either impaired glucose tolerance, or sedentary or trained

(Skov-Jensen *et al.* 2007, Skovbro *et al.* 2008, Vigelsø *et al.* 2013). In the present study, only muscle biopsies from the healthy, but sedentary control participants and the trained participants are studied to elucidate the effect of having a high VO_2max in middle-age on key proteins in lipid and glucose metabolism. Most of the descriptive data on these participants have been reported previously (Skov-Jensen *et al.* 2007, Skovbro *et al.* 2008, Vigelsø *et al.* 2013) and this is clearly referenced in the paper.

Testing and experimental procedure

The study consisted of a screening/test day and an experimental day, both performed after an overnight fast and separated by 4-7 days. On the first day, anthropometrical measures were obtained (body weight and height; body composition (dual-energy X-ray absorptiometry, DXA; DPX-IQ 240, Lunar, Madison, WI, USA)). Peak oxygen uptake, (VO_2max) was determined using a graded exercise test on a semi-supine bike and oxygen consumption, was monitored on an online system (Oxycon Pro system, Jaeger, Hoechberg, Germany).

On the experimental day, muscle biopsies were obtained from *m. vastus lateralis* after 30 min of rest in the supine position using the Bergström muscle biopsy needle technique modified to include suction. The muscle tissue was frozen in liquid nitrogen within 10-15 s of sampling and stored at -80°C until further analysis. Thereafter, the participants underwent a two-step sequential euglycemic-hyperinsulinemic clamp, lasting 120 and 90 min for step 1 and 2, respectively. In step 1 and 2, an insulin infusion rate of 28 and 80 $\text{mU}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$ were used, respectively. The glucose infusion rate (GIR) was adjusted according to frequent measurements (~ 5 min intervals) of blood glucose concentrations (ABL 625, Radiometer, Copenhagen, Denmark).

Western blotting analysis of muscle samples

The procedure has previously been described in detail (Vigelsø *et al.* 2013). In brief, all biopsies were dissected free of visible adipose tissue, connective tissue, and blood. The biopsies were then homogenized in a buffer (25 mM Tris pH 6.8, 2 mM sodium orthovanadate, 5 mM EDTA, 20 mM pyrophosphate, 3 % SDS and 20 mM β -glycerophosphate) heated to 95°C . Protein concentration was measured by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL USA). 20 μg of protein was heated to 55°C for 10 min and electrophoresed in 4-15 % polyacrylamide sodium

dodecyl sulphate gels (Bio-rad, Copenhagen, Denmark) and electro transferred to a PVDF membrane. Membranes were blocked for 1½ h at room temperature with either skimmed milk powder or bovine serum albumin (BSA) diluted in Tris-buffered saline and incubated with primary antibody overnight at 4°C . The primary antibodies were: Antibodies against mitochondrial subunit III-V (Total OXPHOS, ab110411, Abcam, Cambridge, UK), anti-insulin receptor subunit beta (sc-81465, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), anti-Akt I (2967) & II (2964), (Cell Signaling Technology, Beverly, MA, USA), anti-HK I (2024) & II (2867) (Cell Signaling Technology), anti-glycogen synthase (ab40810, Abcam, Cambridge, UK), anti-GLUT4 (PA1-1065, Fischer Scientific, Roskilde, Denmark), anti-LIPG (EL, 07197-1E11, Sigma-Aldrich, Saint Louis, MO, USA), anti-LPL (sc-32885, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), anti-ADRP (Perilipin 2, NB 110-4087, Novus Biologicals, Littleton, CO, USA), anti-TIP47 (Perilipin 3, R04251, Sigma, Prestige Antibodies, St. Louis, MO, USA), anti-oxPAT (Perilipin 5, NB110-60509, Novus Biologicals, Littleton, CO, USA), anti-ATGL (#2138, Cell Signaling Technology) and anti-HSL (sc-74489, Santa Cruz Biotechnology, Inc.). Secondary antibodies were: polyclonal goat anti-rabbit horseradish peroxidase conjugated, (Dako, Glostrup, Denmark) and polyclonal goat anti-mouse horseradish peroxidase conjugated (Dako). The blots were developed in ECL detection reagents (Amersham, ECL Western Blotting Detection Reagents; GE Healthcare) and the chemiluminescence emitted from immune-complexes was visualized with an LAS 3000 image analyzer (FUJI FILM, Tokyo, Japan). The images were quantified by Multi Gauge software (FUJI FILM) and specific signals expressed as a percentage of an internal standard loaded (a pooled sample from all samples) in quadruple on each gel. The results are presented as relative to the average of the sedentary control group.

Biochemical analysis of intramuscular triglycerides

The intramuscular triglyceride content was biochemically determined as previously described (Folch *et al.* 1957, Kiens and Richter 1996). Muscle biopsies were carefully dissected free of excess lipids, connective tissue, and blood. Lipids were extracted using Folch's solution (chloroform 99.8 %, methanol 99.8 % and water in a 1:1:1 ratio) and glycerol was measured photometrically.

Statistics

All statistical analyses were performed in Sigma Plot 12.5 (Systat software, Inc., San Jose, USA). Comparisons of group characteristics and protein expression were made using a Student's t-test. For correlations between different variables Pearson's product moment correlation coefficient (R^2) and corresponding P -value, were obtained. The level of significance was set at $P < 0.05$. Data are expressed as mean \pm standard error of mean (\pm SEM).

Results

Study participants

The characteristics of the participants, some have been published elsewhere (Skov-Jensen *et al.* 2007, Skovbro *et al.* 2008, Vigelsø *et al.* 2013), are given in Table 1. In brief, the TR had lower ($P < 0.05$) body mass index (BMI), weight, and whole body fat content compared with CON. TR had higher ($P < 0.05$) steady state glucose infusion rate (ssGIR) ($\text{mg} \cdot [\text{kg LBM}]^{-1} \cdot \text{min}^{-1}$)

and VO_2max ($\text{ml O}_2/\text{min}/\text{kg LBM}$) compared to CON. Likewise, intramuscular glycogen content was higher ($P < 0.05$) in TR compared to CON (Table 1). Additionally, TR had higher ($P < 0.05$) plasma HDL concentration compared to CON (1.88 ± 0.08 vs. $1.40 \pm 0.07 \text{ mmol} \cdot \text{l}^{-1}$ for TR and CON, respectively) (Skov-Jensen *et al.* 2007). There was no difference in IMTG between the groups (Table 1).

Protein levels

The protein level of mitochondrial complex III and IV was app. 100 % higher in TR compared to CON. Mitochondrial complex V expression was 60 % greater in the TR than in CON (Fig. 1A).

Glucose metabolism

The insulin receptor subunit beta expression tended ($P < 0.08$) to be higher in TR compared to CON, whereas there was no difference in Akt I and Akt II expression (Fig. 1B).

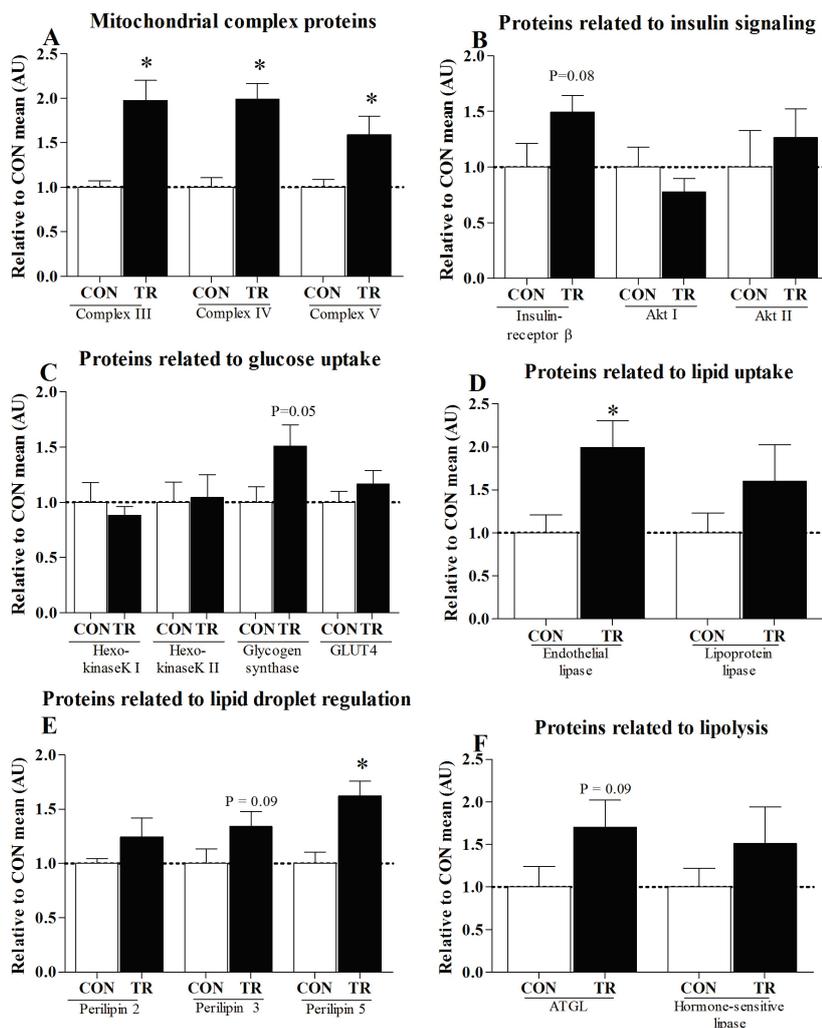


Fig. 1. Protein levels of important lipases in human skeletal muscle. **A)** mitochondrial complex III, IV and V; **B)** insulin receptor subunit α , AKT I, and AKT II; **C)** hexokinase I, hexokinase II, glycogen synthase, and GLUT4; **D)** endothelial lipase and lipoprotein lipase; **E)** perilipin 2, 3 and 5; and **F)** adipose triglyceride lipase (ATGL), and hormone-sensitive lipase was determined by Western blotting in healthy sedentary controls (CON, $n=8$) and endurance trained men (TR, $n=8$). The results are presented as means \pm SEM. The results are normalized against the means of the standard bands ($n=4$), which is a pooled standard sample used for all Western blots in the study (STD). $20 \mu\text{g}$ of muscle homogenate was loaded in all lanes. Representative blots are seen in Figure 3.

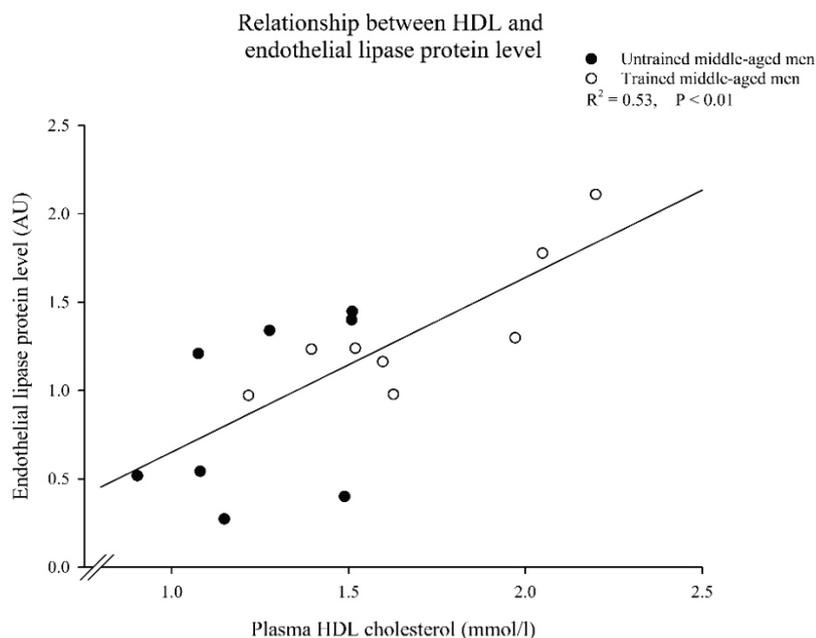


Fig. 2. Relationship between plasma HDL cholesterol and endothelial lipase protein level in endurance trained and untrained middle-aged men. There was a significant positive correlation ($R^2=0.53, P<0.01$). When the correlation was tested separately for the two groups, significance was only observed for the trained group ($R^2=0.55, P<0.05$), but not for the untrained group ($R^2=0.22, P=0.22$), plots not shown.

The glycogen synthase expression tended ($P=0.05$) to be higher in TR compared to CON. However, there was no difference in hexokinase I, hexokinase II and GLUT4 protein levels between the groups (Fig. 1C).

Lipid metabolism

The EL expression was 100 % higher ($P<0.05$) in TR compared to CON, with no difference in the LPL protein level (Fig. 1D). For the lipid droplet proteins the perilipin 5 expression was 50 % higher in TR compared to CON (Fig. 1E). In addition perilipin 3 expression tended to be higher ($P=0.09$) in TR than CON, whereas perilipin 2 expression did not differ between the groups (Fig. 1E). The ATGL expression tended to be higher 70 % ($P=0.09$) in TR compared to CON (Fig. 1F), whereas there was no difference in muscle HSL expression between groups (Fig. 1D). We observed a positive correlation between plasma HDL and protein expression of EL ($P<0.01, R^2=0.53$, Fig. 2). Interestingly, a separate analysis within the two groups revealed that the correlation was mainly driven by TR ($R^2=0.55, P<0.05$) and not CON ($R^2=0.22, P=0.22$) (Fig. 2).

Representative Western blots

For both glucose and lipid metabolism related proteins representative blots of the measured proteins are shown in Figure 3.

Discussion

The main finding is that a high whole-body

VO_{2max} , reflected in higher muscle mitochondrial complex expression, in middle-age is associated with higher muscle protein levels of endothelial lipase and perilipin 5 protein. Furthermore, we observed that plasma HDL concentration correlated positively with the endothelial lipase protein expression. In addition, muscle protein levels of perilipin 3, ATGL, insulin receptor subunit β , and glycogen synthase tended to be higher in the trained compared to the untrained middle-aged men. It is likely that these differences contribute to a higher metabolic flexibility and thus a lowered risk of developing metabolic diseases in the middle-aged trained compared to the untrained men.

Glucose metabolism

As expected based on the literature (Taylor *et al.* 1972), we observed higher muscle glycogen synthase expression consistent with the markedly higher muscle glycogen stores in the trained group. However, it was unexpected that there was no difference between the groups in hexokinase II (Frosig *et al.* 2007), AKT I and II (Frosig *et al.* 2007) and GLUT4 (Dela *et al.* 1993, Hickner *et al.* 1997, Ren *et al.* 1994), since these are normally, at least in young individuals, higher in trained compared to untrained individuals. The insulin sensitivity was as expected markedly higher in the trained compared to the untrained group, and it is thus possible that differences in intrinsic activity and/or in intracellular compartmentalization, possibly coupled to the aging process, rather than the protein level can explain this.

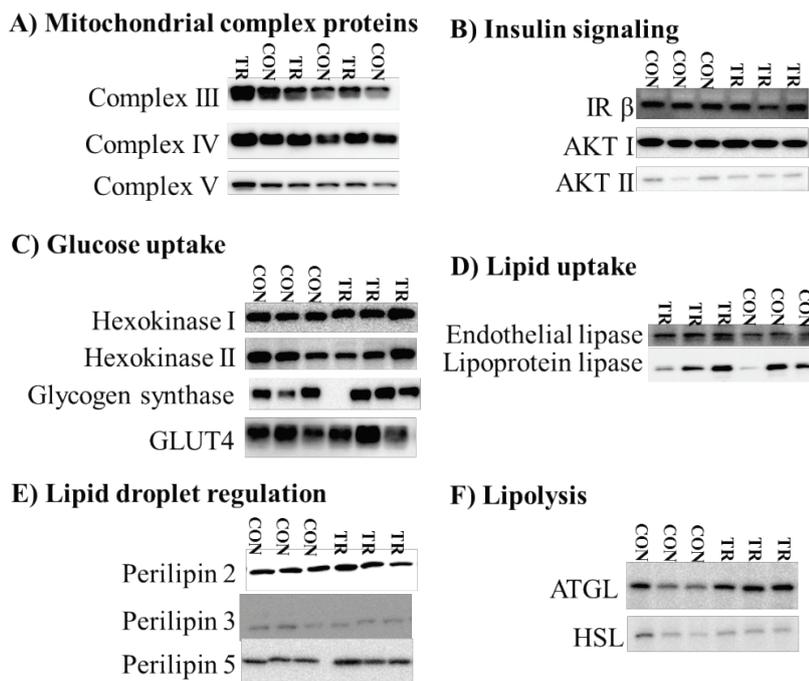


Fig. 3. Representative blots for data presented in Figure 1. **A)** mitochondrial complex III, IV and V; **B)** insulin receptor subunit α (IR β), AKT I, and AKT II; **C)** hexokinase I, hexokinase II, glycogen synthase, and GLUT4; **D)** endothelial lipase and lipoprotein lipase; **E)** perilipin 2, 3 and 5; and **F)** adipose triglyceride lipase (ATGL), and hormone-sensitive lipase (HSL). TR: endurance trained middle-aged men. CON: untrained middle-aged men.

Lipid metabolism

We demonstrate that trained compared with untrained middle-aged men have greater protein expression of EL in the endothelium of skeletal muscle tissue. In a prior study we observed that EL expression was similar between untrained healthy middle aged men and age and lean body mass matched men with type 2 diabetes or impaired glucose tolerance (Vigelsø *et al.* 2013). Not much is known about the expression and function of endothelial lipase in human muscle tissue, but it is likely that a higher capillarization in the trained compared to the untrained muscle contributes to the higher expression in the endothelium of the trained muscle. Interestingly, we observed a positive correlation between plasma HDL and endothelial lipase protein level, which was mainly driven by a positive correlation in the trained but not the untrained group. This is in contrast to prior studies in mice with either whole-body knock out (Ishida *et al.* 2003, Ma *et al.* 2003) or global overexpression (Ishida *et al.* 2003, Jaye *et al.* 1999) of EL. These studies show an inverse relationship between plasma HDL and EL. There is no apparent explanation for this discrepancy, however, species difference, an artifact of the animal models (knock out and over expression) and/or divergent physiological roles for EL in the endothelium of e.g. the liver and adipose compared to muscle tissue may explain this. However, it is well known that regular training increases plasma HDL-concentration (Kiens *et al.* 1980, Nørregaard *et al.* 2015)

and muscle capillarization (Coggan *et al.* 1992, Hepple *et al.* 1997) and this supports the observation of a positive correlation in the trained middle-aged men. Thus, it is possible that EL expression and plasma HDL are influenced by the same training induced mechanism. Although speculative, it is possible that EL could also contribute to FA uptake in skeletal muscle, as it has demonstrated for FA uptake in fat cells in mice (Kratky *et al.* 2005). Overall there is a need for further studies examining the expression and function of EL in human skeletal muscle.

We observed no difference in LPL protein expression, which was unexpected since LPL has been shown to increase with training in young individuals (Lithell *et al.* 1979, Nikkila *et al.* 1978, Taskinen and Nikkila 1980). However, in line with our observation, 6 months of endurance training in middle-aged men did not change muscle LPL mRNA levels (Smith *et al.* 2009). Further studies applying a longitudinal rather than a cross sectional design are needed to further elucidate the effects of training in middle-aged and older men on LPL.

The higher protein levels of perilipin 5 along with a similar trend for higher ATGL and perilipin 3 protein levels indicate that the trained middle-aged men have an improved lipid droplet regulation. The training induced increase in perilipin 5 expression in middle-aged men are in agreement with previously reported data in younger men (Bosma *et al.* 2012, Shepherd *et al.* 2013)

and in older individuals (Amati *et al.* 2011). The trend toward higher ATGL and perilipin 3 after training is also in agreement with prior studies in young individuals (Alsted *et al.* 2009, Louche *et al.* 2013). Bosma and colleagues proposed that perilipin 5 is an important link between the lipid droplets and the mitochondria by showing increased lipid droplet interaction with mitochondria and higher rates of fatty acid oxidation with a 2-fold overexpression of perilipin 5 (Bosma *et al.* 2012). Therefore, perilipin 5 may be of particular importance in the regulation of tissue oxidative capacity (Bosma *et al.* 2012). Overall it seems that increased perilipin 5 expression in both middle-age as observed in this study, and in younger participants, is a consistent adaptation to endurance training.

Perilipin 2 protein expression was not affected by training in the present study, which is in line with previous findings in young men and women after 8-12 weeks aerobic training (Louche *et al.* 2013, Peters *et al.* 2012), but in contrast to another study (Shaw *et al.* 2012) also in young individuals. In the present study there was no difference in IMTG between the two groups (Table 1), which may indicate that perilipin 2 expression may be dependent on IMTG content rather than the training status. This notion is supported by a previously reported association between perilipin 2 and IMTG levels in some (measured by Oil Red O) (Shepherd *et al.* 2012, 2013, 2014), but not all (measured by electron microscopy) (Peters *et al.* 2012) studies. Overall, this highlights that the physiological expression and function of perilipin 2 needs further investigation, particularly since there may be some methodological limitations associated with the biochemical (Steffensen *et al.* 2002) and Oil Red O (Prats *et al.* 2013) analysis methods of IMTG.

Our observation of greater muscle mitochondrial complex II-V protein content is concurrent with the higher VO₂max in middle-aged trained compared to untrained individuals and is consistent with a prior observation from our group (Gram *et al.* 2014).

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Limitations

All the men in the trained group had performed endurance training consistently for many years, but they were not included based on a specific type of training but did various types of endurance training (mainly cycle training and running). Thus, although greater training status, differences in their training regimes, including intensity, duration, and exercise mode as well as their genetic makeup may have influenced their metabolic profile and muscle protein expression.

Conclusion

Our findings underline that having a high VO₂max in middle-age has a favorable effect on key proteins involved in muscle metabolism. This was primarily muscle endothelium lipid metabolism (EL), lipid droplet regulation (perilipin 5) and GS. These adaptations may be favorable for maintaining a high metabolic flexibility, and thus may be important to counter the increased risk of developing metabolic diseases with age.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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